CLONING OF A TYROSINASE GENE IN *STREPTOCOCCUS THERMOPHILUS*

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SUMMARY. The streptococcal cloning vector pIL253 (4.96-kbp, Em^r) was used to introduce the *Streptomyces antibioticus* tyrosinase (*mel*) gene (1.56-kbp) into *S. thermophilus*, an important microbe in dairy fermentations. Electrotransformants of *S. thermophilus* ST128 contained 6.51-kbp recombinant plasmids which probed positively in Southern hybridizations with the biotin-labeled *mel* fragment. Western blots of cell extracts resolved by SDS-PAGE showed the presence of a ca. 31-kDa band thus confirming the synthesis of tyrosinase protein by genetic transformants.

INTRODUCTION

Streptococcus thermophilus is an important representative of a taxonomically diverse group of industrial microbes commonly referred to as lactic acid bacteria (LAB). This group includes streptococci, lactococci and lactobacilli which are essential in many food, agricultural and dairy processes where their main function is the synthesis of acidulants (mainly lactic acid), flavor components and various enzymes. In fermented dairy foods (yogurt and cheeses), thermotolerant *S. thermophilus* is usually co-cultured with lactobacilli. The food-grade status of *S. thermophilus* and the lactobacilli makes them an attractive target as host expression systems for foreign genes. It is therefore not surprising that genetic strain improvement has become a major thrust area in many laboratories around the world. Newly introduced and stably expressed genes would expand the metabolic functions of LAB thus widening the breadth of industrial applications. Recent advances in the field include the synthesis in lactobacilli of heterologous gene products such as endoglucanases (Bates *et al.*, 1989; Baik and Pack, 1990), lipase (Vogel *et al.*, 1990), α-amylase (Jones and Warner, 1990), ß-glucanase (Thompson and Collins, 1991) and cholesterol oxidase (Somkuti *et al.*, 1992), and the transfer and expression of a streptomycete cholesterol oxidase in *S. thermophilus* (Somkuti *et al.*, 1991).

Since there is general interest in natural pigment production in LAB, we have recently investigated the production of melanin-like pigments in *S. thermophilus*, following the introduction of the tyrosinase (*mel*) gene of *Streptomyces antibioticus* (Bernan *et al.*, 1985). In this paper, we report the construction of recombinant plasmids with the *S. antibioticus mel* gene and sP1, a chromosomal promoter sequence of *S. thermophilus*, and the expression of tyrosinase in genetic transformants.

MATERIALS AND METHODS

Microbes and Plasmids

Streptococcus thermophilus ST128 was from our laboratory collection and was cultivated in tryptone-yeast extract-lactose (TYL)

broth (Somkuti and Steinberg, 1986b). *Lactococcus lactis* IL1837, the carrier of pIL253 (4.96-kbp) plasmid with an erythromycin resistance (EmR) marker, was provided by A. Chopin (Institut de Biotechnologie, Jouy-en-Josas, France). *Streptomyces lividans* 1326 harboring pIJ702 (5.6-kbp), the carrier of the *S. antibioticus* tyrosinase (*mel*) gene (Katz *et al.*, 1983) was provided by Y. Murooka (Hiroshima University, Higashi-Hiroshima, Japan) and was grown in brain-heart infusion broth (Difco Laboratories, Detroit, MI).

Plasmid DNA Isolation and Characterization

Plasmid DNA from all bacterial strains was isolated according to a procedure reported earlier (Somkuti and Steinberg, 1986a). Plasmid DNAs were purified by cesium chloride density gradient centrifugation (Stougard and Molin, 1981) and a minicolumn adsorption-desorption procedure (Somkuti and Steinberg, 1986b). Restriction endonucleases were purchased from Life Technologies, Inc. (Gaithersburg, MD) and used according to the manufacturer's instructions.

Agarose gel electrophoresis (AGE) of whole plasmids was done in 0.8% agarose gels (FMC Corporation, Rockland, ME) in TBE buffer (0.089M Tris, 0.089M boric acid, 0.02M EDTA, pH 8.2), at 100 V for 3 h. Restriction fragments were analyzed in 1.2% agarose gels. Molecular weight markers were plasmids of *Escherichia coli* V517 (Macrina et al. 1978) and *Hind*III fragments of λDNA (Life Technologies, Inc.).

Plasmid Electrotransformation

Electrotransformation (ET) followed the general conditions described previously for *S. thermophilus* (Somkuti and Steinberg, 1987). Cells were grown in TYL medium supplemented with 40mM DL-threonine to an OD_{660} of 0.2. Pellets obtained from 4.5 ml of growth medium were washed twice and resuspended in 0.8 ml ET medium (5mM KH_2PO_4, 1mM $MgCl_2{\cdot}6H_2O$, 0.3M raffinose, pH 4.5). After 5 min at 4°C, up to 10μg of DNA in 20μl of ET medium was added and electroporation was performed with a Gene Pulser apparatus (BioRad Laboratories, Richmond, CA) at a field strength of 4kV/cm, and capacitance of 25μF. After electroporation, the ET mixture was immediately mixed with 0.2 ml of 5x TYL medium and incubated for 2 h at 37°C before plating in 10 ml of TYL medium with 1.5% agar. After solidification, the agar film was overlaid with 10 ml of TYL with 1% agar and 15μg/ml erythromycin. Incubation was at 37°C for up to 48 h before scoring transformed clones with EmR phenotype.

Plasmid Constructions

The cloning vector pIL253 (4.9-kbp) was first transferred into *S. thermophilus* ST128 by electrotransformation as described above. After isolation and purification, pIL253 was linearized with *Bam*HI. The streptomycete tyrosinase (*mel*) gene was isolated on a 1.56-kbp fragment after digestion of pIJ702 with *Bcl*I as described by Katz *et al.*(1983) and recovered from agarose gels by electroelution in an Elutrap apparatus (Schleicher and Schuell, Inc., NH). The ligation mixture was at a molar ratio of 3:1 (insert to vector) and ligated with 8U of T4 DNA ligase in 50μl volume at 4°C overnight (Stratagene, La Jolla, CA). The ligation mixture was mixed with 0.8 ml of ET medium (5mM KH_2PO_4, 1mM $MgCl_2{\cdot}6H_2O$, 0.3M raffinose, pH 4.5) and used directly for the electrotransformation of *S. thermophilus* ST128.

Recombinant pMG253 plasmids with a molecular mass of ca. 6.5-kbp were subsequently used as recipients of a segment (sP1) of P25, a *Sau*3AI chromosomal fragment from *S. thermophilus* A054 reported to possess promoter activity (Slos *et al.*, 1991). The 63-bp sP1, flanked by 5'- and 3'- *Eco*RI-*Bam*HI termini, was purchased from Genset Co. (Clifton, NJ).

Southern and Western Analyses

The tyrosinase (*mel*) gene in recombinant plasmids and their restriction endonuclease digests was detected on nylon membranes by Southern hybridizations, with the biotinylated 1.56-kbp *Bcl*I fragment of pIJ702 as the probe. Similarly, the presence of *S. thermophilus* chromosomal promoter sequences in ligation products was detected with the biotin-labeled 45-bp promoter fragment as the probe. Details of

biotin labeling of probes and Southern analysis were described previously (Somkuti *et al.*, 1991).

SDS-PAGE was carried out by the procedure of Laemmli (1970), with the resolving gel containing 10% (w/v) acrylamide.

Western blotting of proteins from sonicated cell extracts after SDS-PAGE was performed essentially by the procedure of Towbin *et al.* (1979). Proteins were transferred from the gel to nitrocellulose membranes using a Bio-Rad Trans-Blot semi-dry transfer system. Immune serum (E. Katz, Georgetown University, Washington, DC, USA) and anti-IgG-horseradish peroxidase conjugate were each used at 1:2500 dilution. Blots were stained with the Sigma *FAST* DAB (3,3'-diaminobenzidine) tablet procedure (Sigma Chemical Co., St. Louis, MO, USA).

Tyrosinase Assays

Tyrosinase activity of sonicated cell preparations was measured by the method of Lerch and Ettlinger (1972). Overnight cells grown in 30 ml of TYL medium supplemented with 0.1mM methionine, 0.02mM $CuSO_4.5H_2O$ and 12μg/ml erythromycin were washed twice with and finally resuspended in 3 ml 50mM K_2HPO_4-KH_2PO_4 buffer pH 7.0 (PO buffer). Tyrosinase activity was measured by mixing 100μl - 500μl of debris-free cell extract with 1 ml of L-DOPA (12mM) in 0.1M PO buffer (pH 6.2), containing 0.2mM $CuSO_4.5H_2O$. Dopachrome (extinction coefficient = 3600) formation was measured at 475 nm in a Beckman DU-70 spectrophotometer for 10 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmole L-DOPA per min. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

RESULTS

Transformation of *S. thermophilus* with pIL253

The 4.96-kbp pIL253, which carries the erthromycin resistance marker derived from pAMß1 (Clewell, *et al.*, 1974) and a multiple cloning site was constructed by Simon and Chopin (1988) as a cloning vector for lactococci. Because of its relatively small size and stability, pIL253 became an attractive candidate for evaluation vector for introducing tyrosinase into *S. thermophilus*. Under electroporation conditions used in this study, pIL253 transformed ST128 to Em^r phenotype at a frequency of 1.7 x 10^3 per μg DNA. Genetic transformants harbored a single plasmid species with a molecular mass of ca. 4.9-kbp. Digestion with *Kpn*I/*Pvu*II, *Kpn*I/*Hind*III, and *Kpn*I/*Bam*HI resulted in anticipated restriction patterns indicating lack of gross deletions or molecular rearrangements of pIL253 in *S. thermophilus*.

Cloning of the mel gene in ST128

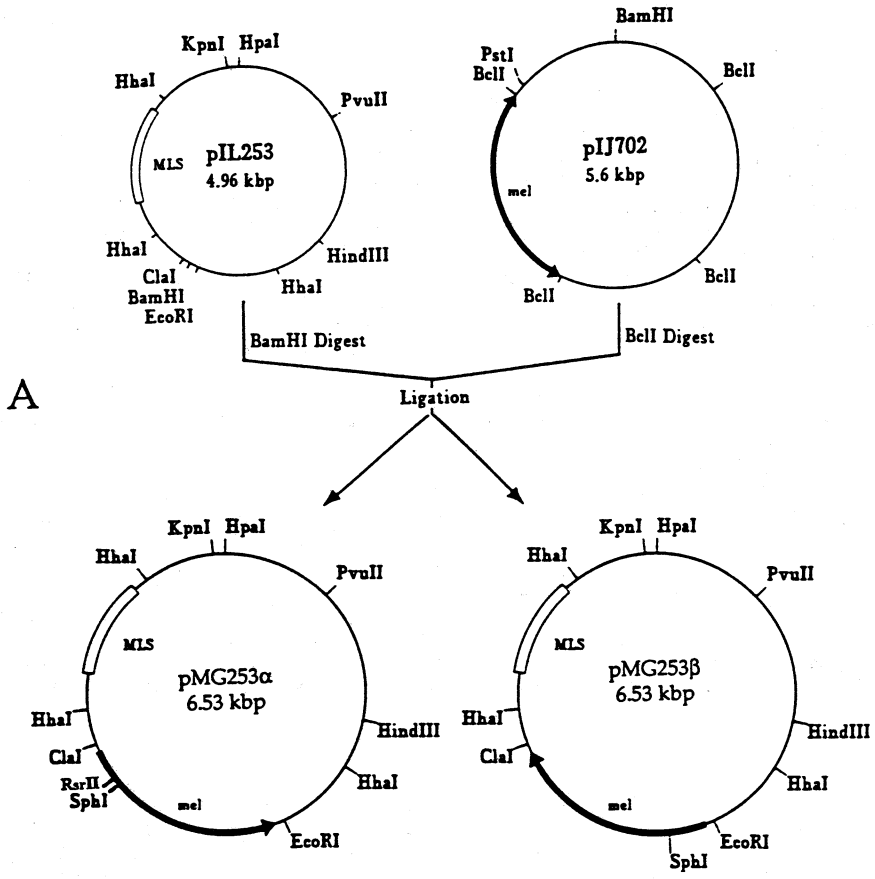
Electrotransformation of ST128 with overnight ligation mixtures of *Bam*HI-linearized pIL253 and the 1.56-kbp *Bcl*I fragment of pIJ702 yielded twenty clones after 48 h of incubation at 37°C. All Em^r transformants carried a single plasmid DNA species, designated as pMG253 (6.53-kbp). Taking advantage of the single asymmetric *Sph*I site located 273 bp downstream from the 5' *Bcl*I terminus of the *mel* gene cluster (Bernan *et al.*, 1985), double digestions with *Kpn*I and *Sph*I identified pMG253α in two transformants in which the *Sph*I site is nearer the *Kpn*I site and pMG253ß in the remaining transformants, in which the orientation of *mel* insert is reversed (Fig. 1A).

Southern hybridizations confirmed the results of AGE (Fig. 1B). The *Kpn*I/*Sph*I digestion of pMG253α yielded two fragments (4.26-kbp and 2.25-kbp) with the larger one giving a strong hybridization signal with the biotinylated 1.56-kbp *mel* probe. Conversely, *Kpn*I/*Sph*I digestion of pMG253ß gave rise to two nearly equal-size fragments only one of which hybridized strongly with the *mel* probe (Fig. 1B).

Once isolated and purified, pMG253α and pMG253ß were as efficient transforming agents as plasmid pIL253, each yielding Em^r transformants at a rate of 1-2 x 10^3 cfu/μg DNA.

Cloning of the *S. thermophilus* chromosomal promoter in pMG253

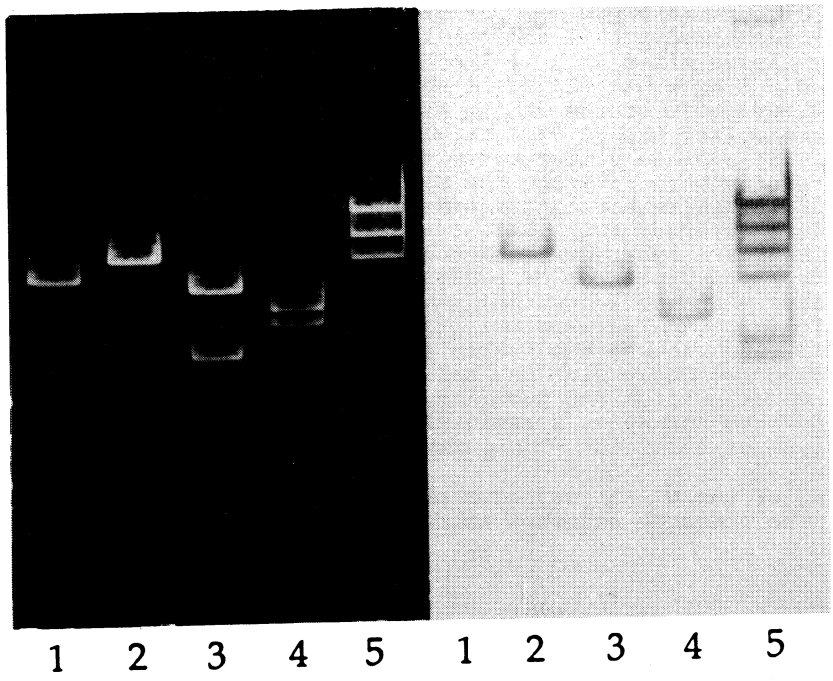
The promoter sequence selected for this study was sP1, an



Legends for Figures

Fig. 1. Introduction of *Streptomyces antibioticus mel* gene into pIL253. A: cloning strategy. B left: AGE digestion patterns of recombinant plasmids (stained with ethidium bromide). Right: Southern hybridization with biotinylated *Bcl*I (*mel*) fragment of pIJ702; Lanes: 1) pMG253/*Kpn*I, 2) pMG253α or ß/*Kpn*I, 3) pMG253α/*Kpn*I-*Sph*I, 4) pMG253ß/*Kpn*I-*Sph*I, 5) *Hind*III fragments of λDNA (in Southern blots, λDNA fragments were visualized with biotin labeled mixture of λ fragments).

B



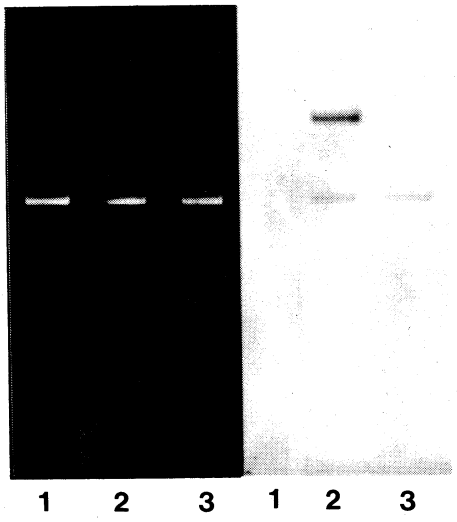


Fig. 2. Agarose gel electrophoresis (left) and Southern blot (right) of recombinant plasmids with *S. thermophilus* sP1 promoter probe. Lanes: 1) pMG253α or ß, 2)pMG253αP; 3) pMG253ßP. Upper bands: oc DNA. Lower bands: ccc DNA.

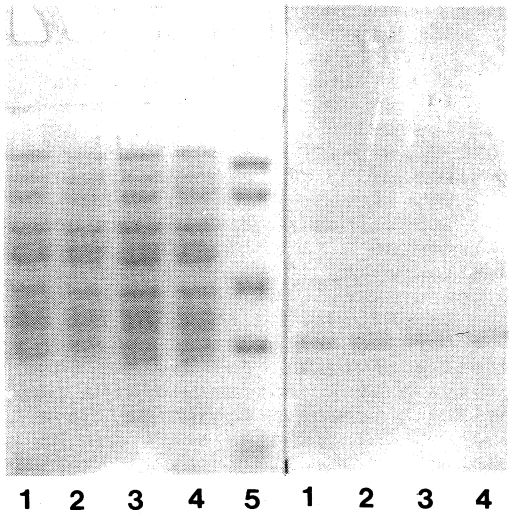


Fig. 3. SDS-PAGE of cell extracts isolated from *S. thermophilus* transformants. Protein components of extracts (stained with (Coomassie Blue, left) and Western blot (right) from pMG253α (1), pMG253αP (2), pMG253ß (3) and pMG253ßP (4). Lane 5: Protein standards in kDa (from top down): phosphorylase b, 94.0; bovine serum albumin, 67.0; ovalbumin, 43.0; carbonic anhydrase, 30.0; soybean trypsin inhibitor, 20.1.

abbreviated form of the 282-bp P25 promoter sequence identified previously in *S. thermophilus* AO54 by Slos *et al.* (1991). The 63-bp sP1 fragment included the putative promoter -35 and -10 regions.

In cloning experiments pMG253α and pMG253ß were linearized with *Rsr*II and *Eco*RI respectively, and sP1 was inserted by blunt-end (pMG253α) or cohesive-end (pMG253ß) DNA ligation protocols. This positioned the sP1 promotor fragment ca. 80 basepairs (pMG253α) and 280 basepairs (pMG253ß) removed from the 5' end of the ORF438 gene in the *mel* fragment. The presence of the promoter sequence in both pMG253αP and pMG253ßP was verified by Southern hybridization (Fig. 2).

Expression of tyrosinase in ST128

The results of Western blot analysis (Fig. 3) indicated the presence of a ca. 31-kDa band in cell extracts of transformants that reacted positively with the tyrosinase antiserum. The putative tyrosinase protein band was present in all genetic transformants harboring either pMG253α, pMG253αP, pMG253ß, or pMG253ßP. Regardless of the presence or absence of the streptococcal promoter sequence sP1, all transformants had low levels of intracellular tyrosinase activity. The amount of L-DOPA oxidized by cell extracts was 0.53 - 1.2 x 10^3 μmole per min and specific activities were calculated to be 0.8 - 1.6 x 10^{-3} units per mg of protein.

None of the *S. thermophilus* transformants produced extracellular tyrosinase, which may explain the lack of melanin-like pigments in the medium. However, when cell free extracts of *mel*$^+$ transformants were incubated with tyrosine (10mM) in PO buffer (pH 6.6) for 3 days at 37°C, the development of yellowish pigmentation was frequently observed.

DISCUSSION

The cloning vector pIL253 showed remarkable stability in *S. thermophilus* and its transformation efficiency by the electroporation process was similar to results found earlier with other plasmids of strptococcal origin (Somkuti and Steinberg, 1988).

Insertion of the 1.56-kbp *BclI* fragment of pIJ702 into pIL253 resulted in pMG253α and pMG253ß, the cell extracts of which contained a tyrosinase-like protein and displayed measurable levels of tyrosinase activity.

Tyrosinase production was not stimulated when the medium was supplemented with methionine, which is an inducer of tyrosinase (Katz and Betancourt, 1988) or copper salt, which was reported to be essential for the synthesis of this monooxygenase (Lerch, 1981).

In pMG253α and pMG253ß, the subsequent insertion of the sP1 (63-bp) streptococcal promoter sequence upstream from the ORF438 portion of the *mel* structural gene did not seem to enhance the expression of tyrosinase by transformants with pMG253αP or pMG253ßP. Possibly the *mel* gene's own promoter region may have to be replaced by sP1 or similar streptococcal promoter sequences to have a significant effect on tyrosinase synthesis in *S. thermophilus*.

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